

Characterization of a T Suppressor Cell Line That Downgrades Experimental Allergic Encephalomyelitis in Mice¹

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A T-suppressor (Ts) cell line of CD8 phenotype was isolated from spleens of SJL/J mice that had recovered from experimental allergic encephalomyelitis (EAE) induced by injection of MBP-activated T cells. The Ts cell line inhibited the proliferation of MBP-sensitized T cells *in vitro*. Addition of recombinant IL-2 enhanced the Ts-mediated suppression. Adoptively transferred Ts line was able to downgrade EAE in mice subsequently challenged with MBP-activated T cells. The mechanism of suppression appeared to involve neither direct cytotoxicity of the effector T cells nor the production of a soluble suppressor factor. The findings suggest an *in vivo* role for suppressor T cells in the regulation of EAE. © 1991 Academic Press, Inc.

INTRODUCTION

Experimental allergic encephalomyelitis (EAE) and chronic relapsing EAE (CREAE) are T cell-mediated autoimmune diseases directed against myelin basic protein (1, 2). CREAE is believed to be a model for multiple sclerosis (3). Although the use of MBP-reactive clones (4) and lines (5, 6) have provided insights into the cellular mechanisms involved in the induction of EAE, the mechanisms involved in its remission has not been fully elucidated. Ts cells have been postulated to play a role in prevention of spontaneous and induced autoimmunity (7, 8). Recovery from EAE in rats (9, 10), natural resistance to EAE induction in certain strains of mice (11), and induction of tolerance by oral route (12-14) are mediated by suppressor T cells. However, less information is available on the role of Ts cells in the modulation of CEAE in the mouse system.

In the present study, we investigated whether a Ts cell line derived from the spleens of SJL/J mice that had recovered from EAE could play a role in the immunoregulation of CREAE *in vivo*. The results indicated that the Ts cell line inhibited both antigen-dependent proliferation *in vitro* and the development of EAE and CREAE *in vivo*, suggesting a role for Ts cells in the regulation of EAE and CREAE.

MATERIALS AND METHODS

Mice. Female SJL/J, 6-8 weeks of age (Jackson laboratory, Bar Harbor, ME), were used in all experiments.

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Immunization. Guinea pig myelin basic protein (GPMBP), purified according to the method of Diebler *et al.* (15), was dissolved in PBS and emulsified with an equal volume of complete Freund's adjuvant (CFA) (Difco laboratories, Detroit, MI) supplemented with 600 $\mu\text{g/ml}$ of lyophilized mycobacterium tuberculosis (TB), strain H37RA (Difco). Each mouse was injected subcutaneously (sc) with a total of 0.1 ml containing 400 μg of MBP and 30 μg of TB. The emulsion was distributed over four sites known to drain the inguinal, axillary, and brachial lymph nodes. Some mice were similarly injected with OVA (Sigma Chemical Co., St. Louis, MO) and CFA as controls.

Induction of EAE. The draining lymph nodes (LN) were aseptically removed 20 days after immunization and lymph node cell (LNC) suspensions were prepared as previously described (16). Briefly, LNs were trimmed of fat, minced, and pressed through 100 mesh stainless steel and washed three times in HBSS to prepare single-cell suspensions. LNC viability was determined by trypan blue dye exclusion and routinely the cells were $>98\%$ viable. The cell concentration was adjusted to $4 \times 10^6/\text{ml}$ and cultured with 50 $\mu\text{g/ml}$ MBP in complete medium (CM), containing RPMI 1640 (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), $5 \times 10^{-5} M$ 2-mercaptoethanol, sodium pyruvate, nonessential amino acids, glutamine, and gentamycin. The cells were cultured in 10 ml volume in 75 cm^2 tissue culture flasks for 96 hr at 37°C . After culture, the MBP-sensitized cells were enriched for T cells by panning technique (17), using petri dishes coated with fetal bovine serum (FBS) to remove adherent cells, washed, and counted, adjusted to $1.5 \times 10^8/\text{ml}$, and 0.2 ml (3×10^7 cells) injected intravenously (iv) via the lateral tail vein into each recipient.

Lymphocyte proliferation. LNC (4×10^5 in 0.2 ml) from primed mice were cultured with 50 $\mu\text{g/ml}$ MBP or 2 $\mu\text{g/ml}$ concanavalin A (Con A; Sigma Chemical Co., St. Louis, MO) in CM in round-bottomed microtiter plates. After 96 hr in culture, the cultures were pulsed with 1 $\mu\text{Ci/well}$ of [^3H]TdR (Amersham Corp., Arlington Heights, IL) for 6 hr. The cells were harvested onto glass fiber filters (Cambridge Technology, Inc. Watertown, MA) with a PHD multiple harvesting unit. In some experiments, LNC from OVA-stimulated mice were also used as controls, in which 50 $\mu\text{g/ml}$ OVA was added to triplicate cultures from both groups of mice.

In vivo suppression of EAE by the Ts cell line. Naive syngeneic recipients were adoptively transferred, iv, via the lateral tail vein with 1×10^7 Ts cells, on the day before (Day -1), on the same day (Day 0), 1 day after (Day +1), 2 days after (Day +2), and 3 days after (Day +3) iv injection of 3×10^7 MBP-activated T cells. All mice were observed daily for clinical symptoms of EAE and graded on a 0-4 scale of increasing severity: 0, no abnormality; 1, flaccid tail with mild hind limb weakness; 2, flaccid tail with moderate hind limb weakness; 3, hind leg paralysis but not complete paralysis; 4, total paralysis and moribund. When a mouse's clinical status remained unchanged for 14 days, it was sacrificed for histopathological evaluation. Mice that did not show any clinical signs of EAE were killed after 6 weeks.

Preparation of spleen cell-conditioned medium. Spleen cell-conditioned medium (SCM) was prepared as previously described (18). Briefly, spleen cells (5×10^6) were stimulated with 5 $\mu\text{g/ml}$ Con A for 48 hr at 37°C . The supernatants were collected and passed through a 0.45- μm membrane filter. Con A was absorbed out of the SCM with Sephadex G-25 (1% W/V for 1 hr at 37°C) and the residual mitogen was neutralized with 0.04 M α -methyl mannoside (Sigma Chemical Co., St. Louis, MO). This SCM

was used as a source of IL-2 for the SCM was quantitated using the

Derivation and maintenance of T cells. Splens of 5 SJL/J mice 29 days post-immunization after the mice had recovered completely were prepared by pressing the minced tissue through a 100 mesh stainless steel and erythrocytes lysed with an ammonium chloride solution ($150 \times 10^6/\text{ml}$ with 50 $\mu\text{g/ml}$ MBP for 96 hr) of macrophages by passage through a 100 mesh stainless steel for 14 days in lectin-free spleen cell-conditioned medium (SCM) containing mitomycin-C (50 $\mu\text{g/ml}$)-treated antigen-presenting cells (APC). At this time, the CD8⁺ T cells were enriched by indirect panning technique (17): T cells were panned on a plate of 100 μm diameter (NY) for 60 min at 4°C . The unbound cells were washed and suspended in PBS containing 1% FBS. The cells were then panned on plates which were coated with goat anti-mouse CD8 mAb for 90 min. As a control the cells were panned on plates coated with anti-CD4 mAb and then panned on goat anti-mouse CD4 mAb. The CD4⁺ cells were rinsed off and the plates were washed with 0.1% EDTA, followed by 0.1% FBS. The cells were maintained in medium supplemented with MBP and APC (Normally ev

Immunofluorescent staining of T cells. T cells were cultured in IL-2 containing medium, washed, and centrifuged to remove debris. Briefly, 1×10^6 cells were panned on a plate of 100 μm diameter (NY) for 30 min at 4°C . The cells were washed with sodium azide. FITC-labeled goat anti-mouse CD4 mAb was added for 30 mins at 4°C . The cells were washed with sodium azide and washed with sodium azide. The cells were stained with FITC-labeled goat anti-mouse CD4 mAb for 30 mins at 4°C . The cells were washed with sodium azide and analyzed by FACS (Cytofluorograph II). The cells were also prepared, air-dried, and stained with FITC-labeled goat anti-mouse CD4 mAb.

Determination of IL-2 activity in culture supernatants. The activity of 5×10^3 CTLL-2 using serial dilutions of culture supernatant activity was compared to a reference standard of maximum response of CTLL-2.

Cytotoxicity assay. Con A-activated T cells were cultured in sodium chromate (^{51}Cr) (Amersham) as previously described (21), followed by 48 hr to allow for the release of excess ^{51}Cr (10^4 cells) in round-bottomed microtiter plates at a ratio of 1:20 and 20:1. The plates were centrifuged at 37°C . After incubation, the plates were washed with water and the tube strips (Skatron Inc. Sterling Heights, MI) were used to absorb the latter and analyzed in a Beckman LS 5500B gamma counter (Beckman Instruments, Inc. Irvine, CA). The results were done in quadruplicates. M

(GPMBP), purified according to PBS and emulsified with an equal volume of Freund's adjuvant (Cappel Laboratories, Detroit, MI) supplemented with casein (sc) with a total of 0.1 ml of emulsion was distributed over four subcutaneous lymph nodes. Some mice (CFA, St. Louis, MO) and CFA as

(N) were aseptically removed 20 days after immunization. Cell suspensions were prepared as follows: mice were killed, removed of fat, minced, and pressed through a 40-mesh sieve into HBSS to prepare single-cell suspensions. Cell concentration was adjusted to 4×10^6 /ml in RPMI 1640 medium (CM), containing RPMI 1640 supplemented with 10% fetal calf serum, sodium pyruvate, nonessential amino acids, and penicillin. Cells were cultured in 10 ml volume in 25-cm² flasks. For MBP-sensitized cells, using petri dishes coated with MBP, cells were washed, and counted, adjusted to 4×10^6 /ml intravenously (iv) via the lateral tail vein.

from primed mice were cultured in 10 ml of medium (Sigma Chemical Co., St. Louis, MO). After 96 hr in culture, the cells were washed, filtered (Cambridge Technology, Cambridge, MA), and counted. In some experiments, cells were cultured in the presence of 50 μ g/ml OVA as an antigen.

naive syngeneic recipients were injected with 1×10^7 Ts cells, on the day after (Day +1), 2 days after (Day +2), or 4 days after (Day +4) MBP-activated T cells. All mice were scored on a 0-4 scale of hind limb weakness; 2, mild hind limb weakness; 3, leg paralysis but not complete; 4, severe paralysis. Mice that died were autopsied. Mice that survived were sacrificed after 6 weeks.

Cells were cultured in lectin-free spleen cell-conditioned medium (SCM). Briefly, spleen cells (5×10^6) were cultured in 10 ml of medium. The supernatants were collected and the cells were washed. The supernatants were absorbed out of the SCM with Sephadex G-10. The medium was neutralized with NaOH. (CFA, St. Louis, MO). This SCM

was used as a source of IL-2 for the expansion of the Ts cell line. The IL-2 activity in the SCM was quantitated using the IL-2-dependent mouse CTLL-2 cell line.

Derivation and maintenance of Ts cell line. The Ts cell line was derived from pooled spleens of 5 SJL/J mice 29 days post adoptive transfer of MBP-activated T cells and after the mice had recovered completely from EAE. Single-cell suspensions were prepared by pressing the minced tissue through stainless steel fine gauze and the erythrocytes lysed with an ammonium chloride lysing buffer. The cells were cultured at 4×10^6 /ml with 50 μ g/ml MBP for 96 hr. The proliferating T cells were then depleted of macrophages by passage through sephadex G-10 column (19) and were expanded for 14 days in lectin-free spleen cell-conditioned medium (SCM), in the presence of mitomycin-C (50 μ g/ml)-treated autologous spleen cells as antigen-presenting cells (APC). At this time, the CD8⁺ (suppressor/cytotoxic) T cells were selected by an indirect panning technique (17): The cells were incubated with a saturating concentration of anti-Lyt 2.2(CD8) mAb (Accurate Chemical and Scientific Corp., Westbury, NY) for 60 min at 4°C. The unbound mAb was washed off and the cells were resuspended in PBS containing 1% FBS. The cells were then allowed to bind to petri dishes, which were coated with goat anti-mouse IgG and the plates were incubated at 4°C for 90 min. As a control the cells were treated with normal mouse IgG in place of the mAb and then panned on goat anti-mouse IgG-coated plates. The nonadherent cells (CD4⁺) were rinsed off and the adherent cells (CD8⁺) were detached by treating the plates with 0.1% EDTA, followed by washing and counting. The CD8⁺ population was maintained in medium supplemented with 10% SCM, with periodic restimulation with MBP and APC (Normally every 15 days).

Immunofluorescent staining of Ts cell line. The Ts line which has been maintained in IL-2 containing medium, was centrifuged over Ficoll-Hypaque to remove filler cell debris. Briefly, 1×10^6 cells were incubated with a 1:20 dilution of the anti-Lyt 1.2 (CD4) and Lyt 2.2 (CD8) mAbs (Accurate Chemical and Scientific Corp., Westbury, NY) for 30 min at 4°C. The cells were washed in PBS containing 2% FCS and 0.1% sodium azide. FITC-labeled goat anti-mouse IgG was added and incubated for a further 30 mins at 4°C. The cells were washed three times with PBS, then fixed with formaldehyde for 10 min and washed, and the cell surface immunofluorescence was assessed by FACS (Cytofluorograph IIs, Orthodiagnosics, MA). Cytofluorescence slides of Ts cells were also prepared, air-dried, mounted, and examined.

Determination of IL-2 activity in SCM. IL-2 activity was assayed on triplicate cultures of 5×10^3 CTLL-2 using serial dilutions as described by Gillis *et al.* (20). The IL-2 activity was compared to a reference standard and the units of IL-2 calculated as 50% of maximum response of CTLL-2 (Gillis *et al.*, 1978).

Cytotoxicity assay. Con A-activated LN blast cells were labeled with 150 μ Ci of sodium chromate (⁵¹Cr) (Amersham Corp., Arlington Heights, IL) for 60 min at 37°C as previously described (21), followed by incubation in culture medium for 30 min to allow for the release of excess ⁵¹Cr. The Ts cell line was added to the blast target (1×10^4 cells) in round-bottomed microtiter plates to give Ts: target ratios of 5:1, 10:1, and 20:1. The plates were centrifuged at 45g for 3 min and incubated for 4 or 18 hr at 37°C. After incubation, the plates were centrifuged at 150g for 10 min and macrowell tube strips (Skatron Inc. Sterling, VA), which separate the cells from the supernatants, were used to absorb the latter and were counted for radioactivity in a Beckman Model 5500B gamma counter (Beckman Instruments, Fullerton, CA). All the experiments were done in quadruplicates. Maximum and spontaneous release of ⁵¹Cr were deter-

mined by adding 100 μ l of 2% Triton X-100 and medium alone to the targets, respectively. In all experiments, the spontaneous release was always less than 10% of the maximum release. The results are expressed as

$$\% \text{ Specific cytotoxicity} = \frac{\text{Test release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100.$$

Generation of Ts factor (TsF). Briefly, 2×10^6 Ts cells were incubated with 1×10^5 APC (mitomycin-c treated spleen cells) in the presence or absence of 50 μ g/ml MBP. To check that the elaboration of a suppressor factor is not the result of APC, the APC alone was also exposed to MBP. After 96 hr incubation, the supernatants were harvested and stored at -20°C until tested. In some experiments, 2×10^6 Ts cells were frozen at -70°C and thawed at 37°C (22). This process was repeated three times and then ultracentrifuged at 10,000g for 60 min. The supernatant was then assayed for TsF.

Assay for Ts and TsF. For Ts assay, 2×10^4 MBP-primed T cells, 5×10^4 APC and 2×10^4 Ts cells (mitomycin-c treated) were cultured with or without MBP in 96-well round-bottomed microtiter plates in a final volume of 200 μ l. To check for the specificity of the suppression, OVA, an irrelevant antigen was added to some cultures. Similarly, OVA-primed T cells were cultured with the Ts in the presence of OVA. After 96 hr in culture, the cells were pulsed with 1 μ Ci [^3H]TdR for the last 18 hr in culture. The cultures were harvested using a multiple cell harvester and the radioactivity was determined by liquid scintillation spectroscopy. To determine the proliferation of the Ts line, 2×10^4 were cultured with APC and MBP as described.

To test for soluble TsF, the non-antigen-stimulated (control), antigen-stimulated, and freeze/thawed cell supernatants concentrated 10-fold by ultrafiltration using B15 amicon concentrator (Amicon, Danvers, MA) were added to the cultures at a concentration of between 10–50% of the final volume of the cultures. The cultures were incubated for 96 hr and processed as described for the proliferative response.

RESULTS

Derivation and maintenance of Ts cell line. The Ts cell line was derived from the spleens of five mice, adoptively transferred with MBP-activated T cells, that had recovered from EAE. The Ts cell line was maintained by a regimen of 14-day restimulation with MBP and feeder cell, followed by a period of IL-2-dependent expansion. Using this approach, the cells could be expanded up to 20 times their number in the course of the 14-day cycle. The cell line was strictly dependent on regular restimulation with MBP, proliferating poorly on SCM alone for a period of 7–14 days.

For the adoptive transfer experiments, the Ts cell line was used after the second antigen stimulation, where adequate numbers of cells could be generated. During these expansion phase, no changes in their characteristics were noted.

Phenotype of Ts cell line. Flow cytofluorometric and cytoentrifuge slide analysis of the Ts showed the Ts cell line stained intensely for the suppressor/cytotoxic (CD8) surface marker (>95%) and were negative for SIg and were negative for the helper/inducer (CD4) marker (data not shown). The expression of the CD8 marker, however, was unstable in long-term culture with the Ts cell line losing the CD8 marker during the 14 days of the IL-2 expansion phase. Activation of the Ts cell line with MBP and feeder cells restored the expression of the CD8 marker.

Specificity of the Ts cell line. The specificity of the Ts cell line for MBP in comparison with an irrelevant antigen, OVA, was tested by proliferation. The results shown in

Antigenic Specificity

Cell cocultures ^a	
EAE LNC	M
EAE LNC	M
EAE LNC	O
EAE LNC + T-suppressor	M
OVA LNC	M
OVA LNC	O
OVA LNC	N
OVA LNC + T-suppressor	O
OVA LNC + T-suppressor	N
OVA LNC + T-suppressor	C

^a LNC (2×10^4 /well) were cocultured with mitomycin-c treated APC in the presence of either MBP (50 μ g/ml), OVA (20 μ g/ml) or T-suppressor (100 μ g/ml). Cells were labeled with [^3H]TdR and counted. Results are expressed as mean \pm SEM.

Table 1 indicate that the Ts line was specific for MBP-specific response and not the OVA response.

Effect of IL-2 on Ts activity. It has been suggested that Ts cells may mediate their activity by absorption of IL-2. To determine the effects of IL-2 on the suppressor activity, the effects of IL-2 on the suppression of Ts-induced suppression, but rather enhancement of Ts-induced suppression, was highly unlikely that suppression of Ts-induced suppression was related to absorption of IL-2 by Ts cells.

Functional characterization of Ts cell line. The functional activity of the Ts cell line was tested by adding various numbers of MBP-primed T cells in the presence of OVA. The results showed that T cell proliferation was observed at Ts cell concentrations of 10^4 – 10^6 cells/ml, causing complete inhibition.

Addition of Exogenous

Culture conditions	Stimulation
LNC	Med
LNC	MBP
LNC + Ts	MBP
LNC + Ts	MBP

Note. LNC (2×10^4 /well) were cocultured with MBP and/or recombinant IL-2 (rIL-2, 50 u/ml) for 96 hr. Cells were harvested, and counted. Values represent mean \pm SEM of quadruplicate cultures. The data is an average of three experiments.

100 and medium alone to the targets, re-
lease was always less than 10% of
essed as

$$\frac{\text{Spontaneous release} - \text{Spontaneous release}}{\text{Spontaneous release}} \times 100.$$

1×10^6 Ts cells were incubated with 1×10^5
the presence or absence of 50 $\mu\text{g/ml}$ MBP.
or factor is not the result of APC, the APC
incubation, the supernatants were harvested
experiments, 2×10^6 Ts cells were frozen
process was repeated three times and then
the supernatant was then assayed for TsF.
 $< 10^4$ MBP-primed T cells, 5×10^4 APC
were cultured with or without MBP in 96-
final volume of 200 μl . To check for the
relevant antigen was added to some cultures.
red with the Ts in the presence of OVA.
with 1 μCi [^3H]TdR for the last 18 hr in
multiple cell harvester and the radioactivity
scopy. To determine the proliferation
APC and MBP as described.

antigen-stimulated (control), antigen-stimulated,
treated 10-fold by ultrafiltration using B15
IA) were added to the cultures at a con-
volume of the cultures. The cultures were
ed for the proliferative response.

RESULTS

The Ts cell line was derived from the
with MBP-activated T cells, that had re-
tained by a regimen of 14-day restimula-
a period of IL-2-dependent expansion.
ended up to 20 times their number in the
strictly dependent on regular restimulation
ne for a period of 7-14 days.

The Ts cell line was used after the second
ers of cells could be generated. During
characteristics were noted.

metric and cytochrome slide analysis
nly for the suppressor/cytotoxic (CD8)
or SIg and were negative for the helper/
expression of the CD8 marker, however,
s cell line losing the CD8 marker during
ivation of the Ts cell line with MBP and
8 marker.

of the Ts cell line for MBP in comparison
l by proliferation. The results shown in

TABLE 1

Antigenic Specificity of T-Suppressor Cell Line

Cell cocultures ^a	Stimulus	Cpm $\times 10^{-3} \pm \text{SEM}$
EAE LNC	Medium	1.3 \pm 0.02
EAE LNC	MBP	98.0 \pm 5.0
EAE LNC	OVA	1.4 \pm 0.01
EAE LNC + T-suppressor	MBP	2.4 \pm 0.09
OVA LNC	Medium	1.2 \pm 0.03
OVA LNC	OVA	78.6 \pm 0.5
OVA LNC	MBP	1.3 \pm 0.02
OVA LNC + T-suppressor	OVA	75.7 \pm 0.2
OVA LNC + T-suppressor	MBP	1.4 \pm 0.02
OVA LNC + T-suppressor	OVA + MBP	83.8 \pm 2.6

^a LNC (2×10^4 /well) were cocultured with mitomycin-c-treated T-suppressor cell line in a ratio of 1:1 in the presence of either MBP (50 $\mu\text{g/ml}$), OVA (20 $\mu\text{g/ml}$), or both. After 72 hr in culture, the cells were labeled with [^3H]TdR and counted. Results are expressed as mean counts per minute (cpm) \pm standard error of the mean (SEM).

Table 1 indicate that the Ts line was specific for MBP, in that it only inhibited the MBP-specific response and not the OVA-specific response.

Effect of IL-2 on Ts activity. It has been reported that some suppressor T cells mediate their activity by absorption of IL-2 (23, 24). To test this possibility, we examined the effects of IL-2 on the suppressor function of the Ts cell line in test cultures. The results in Table 2 indicate that addition of exogenous IL-2 did not overcome the Ts-induced suppression, but rather enhanced the Ts-mediated suppression. Thus, it was highly unlikely that suppression of MBP-driven proliferation by Ts cell line was related to absorption of IL-2 by Ts cells.

Functional characterization of Ts cell line. The suppressive activity of the Ts cell line was tested by adding various numbers of mitomycin-c treated Ts cells to 2×10^4 MBP-primed T cells in the presence of MBP. As shown in Fig. 1, 50% inhibition of T cell proliferation was observed at Ts: LNC ratio of 0.5, with ratios of 2.5 and 5.0 causing complete inhibition.

TABLE 2

Addition of Exogenous IL-2 Augments TS Activity

Culture conditions	Stimulus	[^3H]TdR incorporation (cpm $\times 10^{-3}$) \pm SEM
LNC	Medium	2.1 \pm 0.2
LNC	MBP	207 \pm 11.9
LNC + Ts	MBP	18.9 \pm 4.2
LNC + Ts	MBP + rIL-2	7.1 \pm 1.0

Note. LNC (2×10^4 /well) were cocultured with Ts cell line (1×10^4 /well) in the presence or absence of MBP and/or recombinant IL-2 (rIL-2, 50 u/ml) for 96 hr. The cultures were labeled with 1 μCi [^3H]TdR, harvested, and counted. Values represent mean counts per minute (cpm) \pm standard error of mean (SEM) of quadruplicate cultures. The data is an average of two experiments.

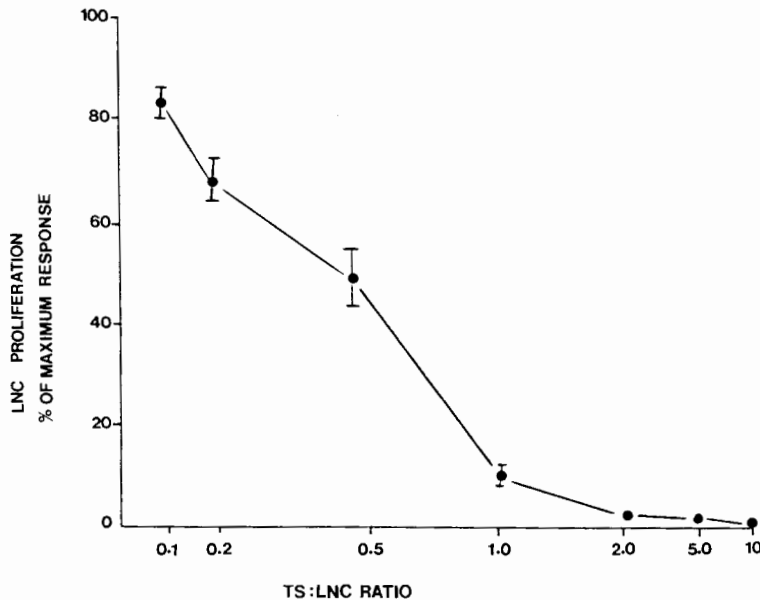


FIG. 1. Inhibition of MBP-driven proliferation of LNC by Ts cell line. Various doses of mitomycin-c-treated Ts cells were added to triplicate cultures of 2×10^4 LNC with or without MBP (50 $\mu\text{g}/\text{ml}$). The proliferation of the LNC was assessed by [^3H]TdR incorporation. Results are expressed as percent (%) of maximal response of LNC plus MBP alone \pm standard error of mean (SEM) ($118.8 \pm 14.0 \times 10^{-3}$ counts per minute, cpm) Background of Ts cells + APC was 0.2×10^{-3} cpm, and background of unstimulated LNC was 4.2×10^{-3} cpm.

In vivo suppression of EAE by the Ts cell line. The ability of the Ts cell line to downgrade EAE was tested *in vivo*. In order to determine the number of suppressor cells required to transfer suppression, groups of naive syngeneic recipients were adoptively transferred *iv* with $1-3 \times 10^7$ Ts cells and then simultaneously challenged by *iv* injection of 3×10^7 (encephalitogenic dose) of MBP-activated T cells in CM. All

TABLE 3

Effect of Suppressor Cell Dose on the Induction of EAE in Mice

Number of Ts cells transferred	Clinical EAE in recipients	
	Incidence	Severity
1×10^7	0/5	0
2×10^7	0/5	0
3×10^7	0/5	0
Control (MBP-activated LNC)	5/5	3

Note. Recipient syngeneic mice were adoptively transferred *ip* with Ts cells ($1-3 \times 10^7$) and simultaneously challenged *iv* with encephalitogenic dose (3×10^7) of MBP-activated T cells. Animals were observed daily for clinical symptoms of EAE. Scoring was on an arbitrary scale of 0-4 as described under Materials and Methods.

TREATMENT	TS LINE TIME BEFORE INJECTION
MBP-PRIMED	
LNC	NONE
LNC	DAY -1
LNC	DAY 0
LNC	DAY +1
LNC	DAY +2
LNC	DAY +3

FIG. 2. *In Vivo* Suppression Of EAE by adoptively transferred *iv* with 3×10^7 MBP indicated. Control animals received MBP EAE. Grading was on the scale of 0 to 4. S and nonprotected groups. The data are th

though significant reduction in transferred mice, as compared to no obvious differences in the extent of Ts cells (Table 3). Thus, a cell In an attempt to determine tively transferred with the Ts ce injection of encephalitogenic do protection of the mice from EA before, on the same day or 1 da of Ts cells after 2 or more days v with CFA + PBS failed to trans spleen or LN. Similarly, heat-ki development of EAE.

Time of cell transfer	Duration of
None	
Ts Cell Line ^b	

^a 3×10^7 cells given *iv*.
^b 1×10^7 cells given *iv*.

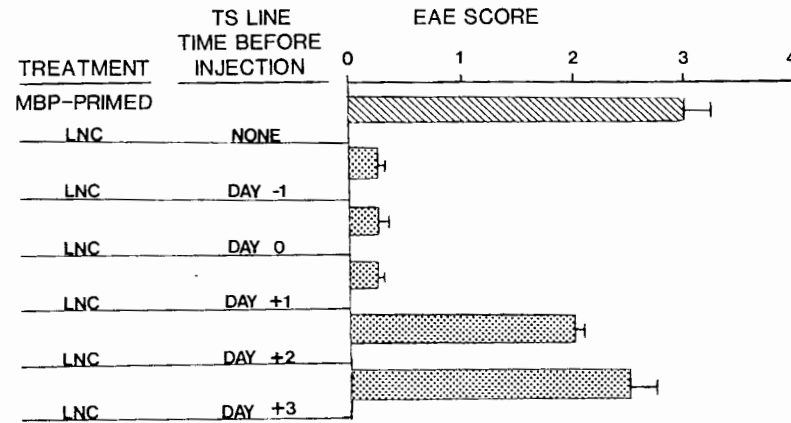


FIG. 2. *In Vivo* Suppression Of EAE by Ts Cell Line. Syngeneic recipient mice, six in each group, were adoptively transferred iv with 3×10^7 MBP-activated T cells. Ts cells (1×10^7) were injected iv on the days indicated. Control animals received MBP-activated T cells only. Animals were observed daily for clinical EAE. Grading was on the scale of 0 to 4. Shown is the mean score of clinical disease \pm SEM in the protected and nonprotected groups. The data are the average of three separate experiments.

though significant reduction in the severity of EAE was observed in the Ts adoptively transferred mice, as compared to MBP-activated T cell injected controls, there was no obvious differences in the extent of EAE suppression in mice given different numbers of Ts cells (Table 3). Thus, a cell dose of 1×10^7 was used in all subsequent experiments.

In an attempt to determine the mode of action of the Ts cell line, mice were adoptively transferred with the Ts cell line at Days -1, 0, +1, +2, and +3 followed by injection of encephalitogenic dose of MBP-activated T cells. As shown in Fig. 2, full protection of the mice from EAE was seen when the Ts cell line was injected 1 day before, on the same day or 1 day after injection of MBP-activated T cells. Injection of Ts cells after 2 or more days was not protective. Donor cells from mice immunized with CFA + PBS failed to transfer suppression irrespective of whether the source was spleen or LN. Similarly, heat-killed Ts cell line failed to protect recipients against the development of EAE.

TABLE 4

Duration of Suppressor Cell Activity in Recipients

Time of cell transfer	Time of challenge ^a (after transfer)	Clinical EAE (incidence)	EAE score
None	MBP-activated T cells	5/5	4
Ts Cell Line ^b	1 week	0/5	0
	2 week	0/5	0
	3 week	2/5	2
	4 week	5/5	4
	8 week	5/5	4

^a 3×10^7 cells given iv.

^b 1×10^7 cells given iv.

cell line. Various doses of mitomycin-c with or without MBP (50 μ g/ml). The Results are expressed as percent (%) of mean (SEM) ($118.8 \pm 14.0 \times 10^{-3}$ counts cpm, and background of unstimulated

the ability of the Ts cell line to reduce the number of suppressor cells. Syngeneic recipients were adoptively transferred simultaneously challenged by MBP-activated T cells in CM. Al-

Incidence of EAE in Mice

Clinical EAE in recipients

Severity

0
0
0
3

Ts cells ($1-3 \times 10^7$) and simultaneously MBP-activated T cells. Animals were observed daily for clinical EAE as described under Materials and Methods.

TS Cell Line	% Specific ⁵¹ Cr release
	18 hr
	1.8
	2.3
	3.2

y (see Materials and Methods for details)

s of mice adoptively transferred
r of EAE. It can be seen in Table
t, since the recipients developed
ized T cells.

ility of MBP carry-over was in-
of the supernatants from freeze/
vious findings with radiolabeled
cells, after 3× washing, would be

L-2 consumption by the Ts cell
nvestigated whether the suppress-
lls. Con A blast LN cell targets
d at 5:1, 10:1, and 20:1 effector

TS Cell Line	Added (cpm × 10 ⁻³) ± SEM
	207.2 ± 11.8
	204.6 ± 8.2
	205.9 ± 9.4
	203.8 ± 8.4
	201.2 ± 8.9
	199.7 ± 1.6
	200.6 ± 7.9
	198.6 ± 4.7
	189.9 ± 8.9
	188.7 ± 1.6
	185.6 ± 7.9
	187.6 ± 4.7

activated or freeze/thawed extract, con-
ious concentrations in the presence of
μCi [³H]TdR, harvested, and counted.
mean (SEM) of quadruplicate cultures.

to target ratios and incubated for 4 or 18 hr. The results are shown in Table 5, and it can be seen that the specific ⁵¹Cr release, in the presence of 20-fold excess of Ts cells did not kill the target cells. Even after 18 hr incubation, there was still no significant release of ⁵¹Cr. Thus, the Ts cells were not acting as classical cytotoxic T cells. We examined the possibility that the suppression was mediated by a soluble suppressor factor. Table 6 shows that neither conditioned medium nor MBP-activated Ts supernatants concentrated 10-fold by ultrafiltration (Amicon, Danvers, MA) caused suppression of MBP-driven proliferation. In some experiments, freeze/thaw extracts of the Ts cells were also tested, but again, no suppression of MBP-driven proliferative responses was seen. The results demonstrate that the suppression was not mediated via a soluble suppressor factor released spontaneously or during the antigen-specific proliferative response.

DISCUSSION

This study was undertaken to elucidate the cellular requirement for the generation of a suppressor T cell line in mice that may be involved in the regulation of EAE *in vivo*. The Ts cell line was derived from the spleens of mice that had recovered from EAE by phenotypic selection after *in vitro* expansion with MBP. The rationale for this methodology was to reduce contamination by helper cells that might become established in the Ts line. The line was able to inhibit MBP-driven T cell proliferation *in vitro*. The suppression appeared antigen-specific since an OVA-specific T cell line in the presence of OVA was not inhibited by the Ts cell line, whereas the MBP-primed T cells in the presence of MBP and the Ts cell line was markedly inhibited (Table 1). The dose-response curve of inhibition of MBP-driven proliferation showed that a high suppressor to MBP-primed T cell ratio was needed for complete suppression, suggesting that not all the cells in the Ts cell line are functionally active suppressor cells. The Ts cell line did not proliferate to MBP in the absence of APC, suggesting that the latter may be required for the induction and expression of Ts activity. There are conflicting reports of whether Ts cells can respond to antigen in the absence of APC (25, 26). The Ts line adhered to MBP-coated wells, suggesting that they possessed idiotypic receptors for MBP. The Ts cell line expressed the CD8 marker, although, in long-term (i.e., 4 weeks) cultures the Ts lines slowly lost the CD8 antigen, while retaining suppressor function. Upon exposure of the Ts cell line to MBP plus APC, the expression of the CD8 antigen was restored. The percentage of cells involved in the cyclic expression of the CD8 antigen has not been well enumerated. It is conceivable that upon activation with MBP, all the CD8⁻ Ts cells may reexpress the CD8 antigen at some stage of proliferation and/or differentiation and that the CD8 antigen may represent some sort of activation marker. This loss of surface antigen in long-term culture is similar to that reported for a Ts cell line in experimental autoimmune uveoretinitis in rats (27) and in keyhole limpet hemocyanin-specific Ts cells (28). The growth properties of the Ts cell line indicated that the line was strictly dependent on MBP for the IL-2 expansion phase, in the absence of which, the cell line would slow down and stop growing after 14 days in SCM alone. The *in vitro* life span of the Ts cell line was 6 months, after which the Ts cells ceased to proliferate.

Since the expression of CD8 antigen does not distinguish suppressor from cytotoxic cells (29), it is possible that the inhibition of the MBP-driven proliferation was due to direct cytolysis of the effector T cells. The negative results obtained in the ⁵¹Cr release

assay argued against that possibility. On the other hand, Sun *et al.* (30) reported a CD8 Ts cell line derived from the spleens of rats that had recovered from EAE that regulated EAE via specific lysis of the encephalitogenic T cell line. These authors, however, used the encephalitogenic T cell line to activate and select for the Ts cells. Thus, the mode of action of a suppressor cell line may be dependent on the manner it is initiated.

Although there are many reports on the murine system which suggest that the final stages of a suppressor T cell pathway involve the elaboration of nonspecific suppressor factors (31, 32), we have not been able to demonstrate any soluble suppressor factor production by the Ts cell line. Thus our Ts cell line is more compatible with MHC-restricted cognate Ts cells which is thought to be distinct from Ts cells that produce suppressor factor (33, 34).

There is evidence to suggest that some suppressor T cells may act by consumption of available IL-2 and that addition of exogenous IL-2 to the cultures could reverse the suppressive effect (23). In the current study, however, addition of IL-2 instead, enhanced the Ts activity suggesting that Ts precursors involved in the suppression of MBP-driven proliferation may require IL-2 for growth and differentiation. This is consistent with a report that indicates that activation of alloreactive Ts precursors require IL-2 (35).

To investigate whether the Ts cell line could downgrade EAE *in vivo*, and also to ascertain whether the cell line exerts its effects at the inductive phase of EAE, or later, the Ts cell line was injected either the day before, on the same day, or Days 1, 2, and 3 after injection of MBP-activated T cells. The Ts cell line was effective in protecting syngeneic normal recipients against the development of EAE. Full protection was seen when 1×10^7 Ts cells were injected the day before, on the same day or 1 day after injection of MBP-activated T cells. When the Ts cells were injected 2 or more days after injection of MBP-activated T cells, or after establishment of EAE, they were unable to confer protection. It has been shown in the rat model of EAE (36) that pathogenic MBP-reactive cells break through the blood-brain barrier within 24 hr and proceed to interact with CNS cells, culminating in the inflammatory reaction of EAE. Our findings indicate that suppressor cells injected 2 or more days after the transfer of MBP-reactive T cells may arrive at the CNS too late to curtail the initiated inflammatory response. Thus, the Ts cells apparently act at the inductive phase of the disease process. A similar finding of action of Ts cells in the regulation of EAE in the rat system was previously reported (30). Although iv injections of soluble MBP elicited a transient resistance to EAE, no suppressor cells were isolated. Thus, the inhibition of EAE by soluble MBP might be due to a receptor blockade (37). Resistance to EAE, after induction of oral tolerance to MBP, was adoptively transferred with Con A-activated CD8⁺ cells from either the spleen or mesenteric LN of MBP-fed animals (12). T cells, from these mice suppressed proliferation of MBP-primed LNC *in vitro*. Although oral tolerance to MBP is thought to be mediated, in most studies, by active suppression mechanism, it is also possible that, under some circumstances, oral tolerance to MBP is mediated by clonal deletion (13, 14).

There was no evidence that MBP carry-over was responsible for the suppression of EAE obtained with the CD8 cell line. The Ts cell line must be viable in order to transfer resistance, since heat-killed Ts cell line did not confer protection.

The data presented here suggest the mice from EAE. Studies are in progress of action of Ts cells in the regulation

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other hand, Sun *et al.* (30) reported a rat line that had recovered from EAE that had a different T cell line. These authors, in order to activate and select for the Ts cells, the T cell line may be dependent on the manner

of the immune system which suggest that the final mechanism of elaboration of nonspecific suppressor cells demonstrate any soluble suppressor factor. The T cell line is more compatible with MHC class II, which may be distinct from Ts cells that produce

Suppressor T cells may act by consumption of IL-2. The addition of IL-2 to the cultures could reverse the effect. However, addition of IL-2 instead, enhanced the effect involved in the suppression of MBP synthesis and differentiation. This is consistent with the idea that alloreactive Ts precursors require

to be downgraded EAE *in vivo*, and also to be downgraded at the inductive phase of EAE, or later, or even before, on the same day, or Days 1, 2, and 3. The T cell line was effective in protecting against the development of EAE. Full protection was seen before, on the same day or 1 day after the T cells were injected 2 or more days before or after establishment of EAE, they have been shown in the rat model of EAE to cross through the blood-brain barrier within 24 hours, culminating in the inflammatory response. Suppressor cells injected 2 or more days before arrive at the CNS too late to curtail the effect of the T cells apparently act at the inductive phase. The mechanism of action of Ts cells in the regulation of EAE is reported (30). Although *iv* injections of T cells into EAE, no suppressor cells were isolated. The effect of MBP might be due to a receptor-mediated effect of oral tolerance to MBP, was CD8⁺ cells from either the spleen or lymph nodes. Cells from these mice suppressed proliferation of T cells. Oral tolerance to MBP is thought to be mediated by a receptor-mediated mechanism, it is also possible that the effect of oral tolerance to MBP is mediated by clonal deletion of T cells.

The T cell line was responsible for the suppression of EAE. The T cell line must be viable in order to be effective. It did not confer protection.

The data presented here suggest that Ts cells play an active role in the recovery of mice from EAE. Studies are in progress to determine more precisely the mechanism of action of Ts cells in the regulation of EAE.

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